

## THE INTERACTION OF ALDEHYDES WITH COLLAGEN

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SUMMARY

The interaction of a series of aldehydes with collagen has been examined. Substitution of amino groups, as assessed by the formol titration, varies with the aldehyde and is greatest with formaldehyde, acrolein, succinaldehyde and glutaraldehyde and least with propionaldehyde and adipaldehyde. Except with formaldehyde, glyoxal and dialdehyde starch there is a corresponding decrease in lysine and hydroxy-lysine recoverable on hydrolysis, suggesting that the bond formed is relatively stable. Most of the amino groups reacting appear to be involved in cross-links.

Losses of lysine are accompanied by increases in ultraviolet absorption of the hydrolysates with, in some cases, a maximum at 265 m $\mu$ . With glutaraldehyde three distinct ultraviolet absorbing fractions can be separated on Sephadex G-10. One of these appears to be a compound containing two lysine and six glutaraldehyde residues.

Glyoxal and other compounds containing adjacent carbonyl groups reacted with guanidyl groups of arginine under acid conditions. One primary product is formed in the cold which on heating in 6 M HCl gives a second ninhydrin positive compound.

The implication of the results with respect to the natural cross-links in collagen is briefly discussed.

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## INTRODUCTION

The interaction of formaldehyde with collagen and other proteins has been investigated by numerous workers (see for example, FRENCH AND EDSALL<sup>1</sup> and FRAENKEL-CONRAT and co-workers<sup>2-4</sup>). Less information is available on the other aldehydes though recently MILCH<sup>5-7</sup> has given some attention to those of metabolic interest. Interest has also centred on glutaraldehyde because of its efficiency as a tanning agent and as a fixative in electron microscopy. In work primarily directed towards the cross-linking and stabilisation of collagen (CATER<sup>8,9</sup> and BOWES AND CATER<sup>10</sup>) it became clear that the aldehydes differed considerably in their ability to cross-link collagen both with respect to the number of cross-links introduced and their

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Abbreviation: Z-, benzyloxycarbonyl group.

stability. A more detailed study of the interaction of a series of aldehydes was, therefore, undertaken in order to gain more information on the mechanism of the reactions involved and to try and establish the reasons for these differences. In view of current hypotheses regarding the part played by aldehydes in the maturation of collagen<sup>11,12</sup> and the reported occurrence of Schiff's bases<sup>15,16</sup> in other proteins, such knowledge is of particular interest at the present time.

## MATERIALS AND METHODS

### *Collagen preparation*

A commercial collagen powder (Standard Hide Powder, Baird and Tatlock Ltd., London) was used throughout. This material is prepared from oxhide; a short treatment in dilute alkali and sulphide is given to remove hair and non-collagenous proteins, followed by neutralisation, washing and solvent extraction to remove lipid. Total nitrogen on moisture-free weight was 18.0% and hydroxyproline, 14.1%. For the sake of convenience this preparation is referred to throughout as collagen.

### *Aldehydes*

Glutaraldehyde was obtained from Union Carbide in the form of a 25% solution. Malonaldehyde was prepared from its tetraethyl acetal (Aldrich Chemicals) and succinaldehyde from diethoxytetrahydrofuran (Koch-Light) by acidification with 0.5 M  $\text{H}_2\text{SO}_4$ . Adipaldehyde was prepared by oxidation of cyclohexanediol (Robinson Bros. Ltd., West Bromwich) with sodium periodate at pH 5.0. The reaction mixture was saturated with NaCl, the aldehyde extracted with ether and the ether removed under vacuum with minimum heating. Dialdehyde starch was a 93% oxidised product (Sumstar 190) marketed by Miles Chemical Co., Indiana. Other aldehydes were of laboratory reagent grade obtained from British Drug Houses or Koch-Light.

### *Reaction of aldehydes with collagen*

The collagen was soaked in water overnight, the excess water decanted off and a solution (20 ml per g) of the aldehyde at pH 3 to 4 added. After 1 h the pH was raised gradually, usually to between 7.5 and 8.0, by the addition of  $\text{NaHCO}_3$ . Unless otherwise stated the treatments were for 24 h at laboratory temperature with intermittent shaking.

### *Reaction of glutaraldehyde with amino acids and polylysine*

1 ml of a solution containing 0.2 mmole of the amino acid was mixed with 2 ml of a solution containing 0.8 mmole glutaraldehyde. 5 ml of 5%  $\text{NaHCO}_3$  were added to bring the pH to between 7 and 8 and the reaction allowed to proceed for 2 h.

To 20.9 mg polylysine (= 0.1 mmole lysine) dissolved in 5 ml water was added 0.2 mmole glutaraldehyde dissolved in 4 ml water. The pH was adjusted to 8.0 and a yellowish-red precipitate was formed. After 24 h an equal volume of conc. HCl was added and the whole hydrolysed for 48 h at 105°. Excess acid was evaporated off and the residue made up to 10 ml. A similar mixture of glutaraldehyde and polylysine was hydrolysed directly without any adjustment to pH 8.0.

*Reaction of glyoxal and other dicarbonyl compounds with arginine or carbobenzoxy-arginine*

5 mmoles arginine or *Z*-arginine were reacted with 5 mmoles glyoxal in 20 ml water at pH 8.0 for 24 h at 20° or in 20 ml 12 M HCl for 16 h at 20°.

Aliquots of the solutions were hydrolysed in 6 M HCl for 24 h, together with a freshly mixed solution of the two reactants.

In a further experiment arginine (5 mmoles) was reacted with 10 mmoles glyoxal, methylglyoxal, malonaldehyde or diacetyl at pH 2.0 for 2 and 14 days at 20°.

*Formol titration*

The formol titration has been modified for use with insoluble proteins, the difficulty of obtaining equilibrium in a two-phase system being reduced by the use of pH stat titrator (Radiometer TTT1c)<sup>17</sup>.

In the present investigation 2 g samples of the modified collagen in powder form were titrated in 4% (w/v) NaCl. Titration at each stage was to pH 9.0 over a period of 3 h. The original collagen was titrated under similar conditions and the number of  $\epsilon$ -amino groups substituted obtained by difference. In this way a number of errors inherent in the method are eliminated. Dilution by bound aldehyde will tend to give low results for free amino groups in the modified protein and hence, an overestimate of the degree of interaction. However, the amounts of aldehyde rarely exceed 10% and, in view of the other approximations, it was considered that little advantage would be gained by making nitrogen determinations to correct for this.

*Amino acid analysis*

Samples were hydrolysed in 6 M HCl for 20 h at 105°. Hydrolysates were evaporated on a water bath to remove HCl and made up to a suitable volume. Samples were then analysed using a Bender & Hobein automatic analyser and elution systems described by HANNIG<sup>18</sup>. A modified elution system on 20 × 1 cm IR 120 column (20–25  $\mu$  resin) at 57° with 0.38 M citrate buffer, pH 4.46, was used for the separation of the basic amino acids (M. J. GLIMCHER, personal communication). Hydroxyproline was determined directly on the hydrolysates by a modification of the STEGEMANN<sup>19</sup> method adapted for use on the Technicon Autoanalyser<sup>20</sup>. Results were calculated in terms of amino acid nitrogen as per cent total nitrogen of the hydrolysate and converted to mmoles per 100 g of the original collagen (nitrogen content 18.0%).

*Other analytical methods*

Formaldehyde in modified collagen was determined by steam distillation from 1 M H<sub>2</sub>SO<sub>4</sub> and precipitation with dimedone<sup>21</sup>, and glutaraldehyde in solution by formation of the bisulphite complex and titration with iodine<sup>22</sup>.

RESULTS

*Interaction of aldehydes with the  $\epsilon$ -amino groups of collagen*

The main object of the present investigation was comparison of the reactivity of different aldehydes and treatments were, therefore, in the main carried out under optimum conditions for reaction, *i.e.* excess aldehyde at pH 8.0 for relatively long periods.

Direct chemical determination of aldehyde bound by quantitative release from the collagen was only possible with formaldehyde. Results confirm earlier findings of BOWES AND KENTEN<sup>23</sup>, namely increase in uptake from almost zero at pH 3.0 to the equivalent of 1:1 molar ratio with amino groups at pH 8.0. In contrast, limited work with <sup>14</sup>C-labelled glutaraldehyde showed that appreciable amounts of this aldehyde were bound at pH 4.0, and at pH 8.0 a 1:1 ratio with amino groups was greatly exceeded<sup>24</sup>. Similar conclusions were also reached by FEIN *et al.*<sup>22</sup> who estimated uptake from losses of aldehyde from solution. Under favourable conditions using high concentrations of aldehyde, amounts approaching 100 mmoles per 100 g collagen, equivalent to 3 aldehyde residues per amino groups are taken up.

Provided certain precautions are taken the formol titration can give a useful estimate of the number of amino groups substituted. Results so obtained are given in Table I.

TABLE I

AMINO GROUPS SUBSTITUTED AND CROSS-LINKS INTRODUCED INTO COLLAGEN BY ALDEHYDES

Aldehyde	Conditions of treatment		Amino groups reacted (moles per 10 <sup>5</sup> g)		Cross-links* (moles per 10 <sup>5</sup> g)	Shrinkage temperature (°C)
	pH	% aldehyde on dry wt.	from formol titration	from loss of lysines		
Formaldehyde	8.0	20	32	0	5	87
Propionaldehyde	8.0	6	1.5	3.9	—	—
Propionaldehyde	10.0	6	2.8	—	—	62
Acrolein	8.0	17	27.4	—	11	79
Acrolein	10.0	17	28.9	32.7	—	—
Methylglyoxal	8.0	5	6.0	9.3	—	69
Methylglyoxal	8.0	15	8.7	—	—	—
Glyoxal	8.0	10	16.3	2.5	8	83
Glyoxal	8.0	16	13.6	—	8	83
Malonaldehyde	8.0	18	12.7	10.6	5	71
Succinaldehyde	8.0	2.5	27.1	—	—	—
Succinaldehyde	8.0	10	30.0	21.5	10	73
Glutaraldehyde	4.0	12	16.9	—	7	—
Glutaraldehyde	6.5	12	23.8	—	13	86
Glutaraldehyde	8.0	12	25.0	26.1	12	87
Glutaraldehyde	10.0	12	26.6	—	—	—
Adipaldehyde	8.0	8	4.2	—	2	60
Dialdehyde starch	8.0	20	24.8	6.9	5	75

\* Cross-links introduced into kangaroo tail tendon—taken from CATER<sup>8,9</sup>.

On the basis of earlier evidence it is reasonable to assume that reaction with formaldehyde is complete. With the monofunctional propionaldehyde it is very much less. With the dialdehydes reactivity increases in the order adipaldehyde < malonaldehyde < glyoxal < glutaraldehyde ≤ succinaldehyde. Methyl glyoxal is appre-

ciably less reactive than glyoxal, while acrolein is similar in reactivity to succinaldehyde and glutaraldehyde. With succinaldehyde the number of amino groups substituted approached the maximum but with glutaraldehyde the number reacted never exceeded 26–27 mmoles out of a possible 32–33 even when the temperature was raised or the time of reaction prolonged. Raising the pH of the reaction from 8 to 10 caused only a slight increase in number of amino groups substituted (followed with propionaldehyde, acrolein and glutaraldehyde only).

Except with formaldehyde, glyoxal and dialdehyde starch losses of lysine and hydroxylysine ran parallel with substitution of amino groups as estimated by the formol titration. With glutaraldehyde this agreement was confirmed in additional experiments. Owing to the low degree of interaction in the first place, it is impossible to say with certainty whether propionaldehyde and adipaldehyde behave similarly to formaldehyde or to the higher aldehydes.

Full amino acid analyses were carried out on collagen treated with formaldehyde, glyoxal, malonaldehyde, glutaraldehyde and dialdehyde starch. With formaldehyde there was no significant change except for complete loss of tyrosine. With malonaldehyde and glutaraldehyde, in addition to losses of lysine and hydroxylysine, there was some loss of tyrosine. No additional ninhydrin positive peaks or increase in existing peaks corresponding to the losses of lysine and hydroxylysine could be detected. With glyoxal and dialdehyde starch there were extensive losses of arginine. Appreciable amounts of ninhydrin positive material were eluted overlapping lysine and hydroxylysine and the peaks corresponding to proline, alanine and, to a lesser extent, glycine were increased. With methylglyoxal there was also loss of arginine and additional ninhydrin positive material eluted near lysine. The reaction of these aldehydes with guanidyl residues is considered further on p. 348.

#### *Absorption of ultraviolet light*

Hydrolysates of the modified collagens showed increased ultraviolet absorption compared with corresponding hydrolysates of the original collagen or the individual aldehydes (Fig. 1). With glutaraldehyde there was a definite maximum at 265 m $\mu$ , with malonaldehyde and acrolein there was an indication of such a peak but with succinaldehyde and methylglyoxal there was only an indication of a plateau over the range 255–290 and 255–340 m $\mu$ , respectively.

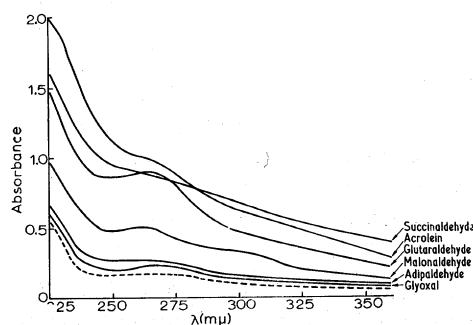


Fig. 1. Ultraviolet spectra of hydrolysates of collagen treated with different aldehydes compared with that of collagen (concn. 0.8 mg collagen per ml). (Unicam SP 800, 1-cm cells.)

KORN AND FILACHIONE<sup>25</sup> have also observed the 265-m $\mu$  maximum with glutaraldehyde and have suggested that it might be used for the determination of glutaraldehyde.

#### *Cross-links*

Cross-links introduced into kangaroo tail tendon by treatment under comparable conditions are also included in Table I. These are taken from papers by CATER<sup>8,9</sup> and were determined by application of the Flory-Rehner equation to stress-strain measurements on the denatured tendon. With glyoxal, malonaldehyde and glutaraldehyde most of the amino groups which have reacted appear to be involved in cross-links. Only with formaldehyde and dialdehyde starch does there appear to be appreciable reaction with single amino groups.

Shrinkage temperatures determined on pieces of skin treated along with collagen powder do not appear to be directly related to the number of cross-links introduced. This may be due to the different type of tissue used, *i.e.* skin as opposed to tendon, or possibly to variations in the stability of the cross-links under the conditions of the two determinations, *i.e.* slow heating at 2° per min compared with heating at 96–100° for 2 min.

#### *Further observations on the reaction of glutaraldehyde with amino groups*

Reaction of glutaraldehyde with polylysine at pH 8.0 led to the formation of yellowish-red precipitate and recovery of lysine following hydrolysis was reduced to 23%. The ultraviolet spectrum of the hydrolysate was similar to that obtained with collagen treated with glutaraldehyde and the absorbance at 265 m $\mu$  in the two preparations was proportional to the number of amino groups reacted in each. Direct hydrolysis of glutaraldehyde and polylysine without a reaction period at pH 8.0 led to no loss of lysine and no increase in ultraviolet absorbance.

Reaction of glutaraldehyde with *N*<sup>α</sup>-Z-lysine,  $\epsilon$ -aminocaproic acid, glycine and alanine also led to increases in ultraviolet absorption, but with these compounds there was only a faint indication of a peak at 265 m $\mu$ . With glycine and alanine the increases in ultraviolet were appreciably less than with the other two compounds, the proximity of the carboxyl group presumably reducing the reactivity of the amino group. On heating the Z-lysine and aminocaproic acid derivatives in 6 M HCl at 105° for 24 h the ultraviolet absorption was doubled indicating a further reaction catalysed by acid but, since it occurred with both compounds, apparently not involving further condensation with  $\alpha$ -amino groups. The absorbance per mole of amino groups was of the same order as that of hydrolysates of the glutaraldehyde-polylysine and collagen reaction products.

The general inference is that increases in ultraviolet absorption are directly related to interaction of glutaraldehyde with amino groups and that secondary condensation with the  $\alpha$ -amino groups is not involved.

Further evidence for the relationship between ultraviolet absorbance and substitution of amino groups was obtained from the examination of collagen treated with increasing amounts of glutaraldehyde (Fig. 2). Both ultraviolet absorbance and loss of lysine residues increased steadily with increasing uptake of aldehyde (as estimated by loss from solution) and appear to be approaching a limit at about 10–12% glutaraldehyde. Between 4 and 5 moles of glutaraldehyde are taken up for every lysine

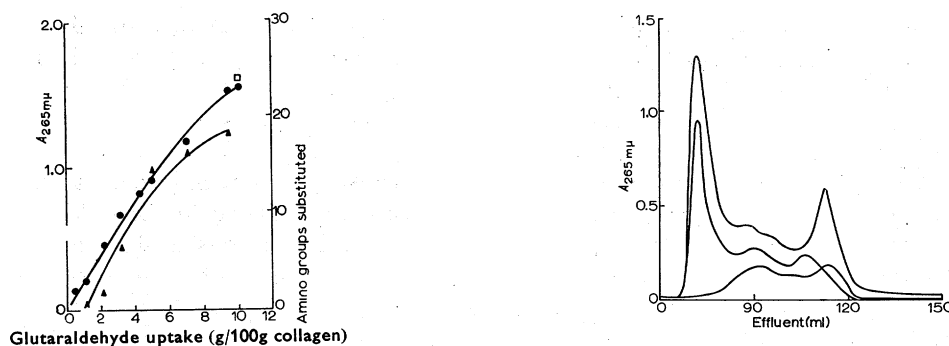


Fig. 2. Absorbance at 265  $m\mu$  of hydrolysates and number of amino groups substituted at increasing levels of glutaraldehyde uptake at pH 8.0 as assessed by determination of losses from solution. Absorbance measured at concentrations equivalent to 1.6 mg collagen per ml hydrolysate in 1-cm cells. ●—●, absorbance at 265  $m\mu$ ; ▲—▲, amino groups substituted; □, absorbance at 265  $m\mu$  after extraction with dioxan. Amino groups substituted expressed in mmoles/100 g collagen.

Fig. 3. Separation on Sephadex G-10, 30  $\times$  2.1 cm column,  $V_0$ , 66.5 ml; elution with water. Lower curve, collagen, untreated, loading 32 mg; middle curve, collagen, treated with glutaraldehyde, loading 32 mg; upper curve, polylysine treated with glutaraldehyde, loading equivalent to 10  $\mu$ moles lysine.

residue lost. However, not all of this is firmly bound since extraction with dioxan, in which polymers of glutaraldehyde are reported to be soluble<sup>26</sup>, removes appreciable amounts of material giving an aldehydic reaction without affecting the ultraviolet absorption. General consideration of the relationship between ultraviolet absorbance and  $\epsilon$ -amino groups substituted indicates a molar absorbance coefficient in terms of substituted  $\epsilon$ -amino groups of about  $5 \cdot 10^3$ .

With the glutaraldehyde treated collagen no new ninhydrin positive compounds were detected during amino acid analysis. However, with a heavy loading of the hydrolysed polylysine derivative, an appreciable amount of a dark coloured material was detected at the top of the 20  $\times$  1 cm IR 120 column. The top layer of resin was removed, the absorbed material eluted with ammonia and examined by thin-layer chromatography on Kieselgel using *n*-propanol-34% aqueous ammonia (67:33, by vol.) for development. A brownish-purple ninhydrin spot was observed just removed from the origin.

Attempts were made to separate the ultraviolet absorbing components of hydrolysates of glutaraldehyde treated collagen and polylysine by molecular sieve chromatography on Sephadex G-10. On a column 30  $\times$  2.1 cm and elution with water the bulk of this material was contained in three peaks, the general pattern being the same for both preparations (Fig. 3). The first peak was eluted with the void volume (66.5 ml) indicating material of molecular weight greater than 700.

Using a larger column (90  $\times$  2.5 cm;  $V_0$ , 170 ml) it was possible to separate sufficient amounts of the material constituting the first peak to enable further examination of the product to be made. The material was insoluble in organic solvents and attempts to purify by recrystallisation were unsuccessful. Infrared examination using the KBr disc technique gave a complicated spectrum serving only to indicate absence of carbonyl groups and the probable presence of amino and hydroxyl groups. Elemental analysis gave a composition of 52.0% C, 7.05% H, 5.48% N, 25.81% C and 9.65% Cl.

and suggests a ratio of three glutaraldehyde residues per lysine residue. A compound containing two lysine residues and six glutaraldehyde residues with a molecular weight of about 900 seems a likely possibility. This fraction is approx. 4% by weight of the treated collagen, thus, on the basis of 2 moles lysine per molecular weight 900, it would account for 8.8 mmoles lysine (or hydroxylysine) out of the 32 present in 100 g collagen, or rather more than one third of the 26 mmoles substituted. The first fraction also represented about one third of the total ultraviolet absorbance of the sample.

TABLE II

ANALYSIS OF ALDEHYDE-TREATED COLLAGENS ON 20 × 1 cm IR 120 COLUMN

The results are expressed in mmoles per 100 g.

	Lys	Hyl	His	Arg	X*	Y*	NH <sub>3</sub>
Original collagen	28.8	6.1	4.2	42.1			22.0
<i>Glyoxal treatment</i>							
Collagen	28.1	4.3	2.6	11.2	14.1	23.1	34.7
Deaminated collagen	2.0	0.0	2.0	12.4	9.9	20.8	43.9
Guanidated collagen	6.5**	0.0	1.6	11.1	10.0	21.6	30.7
<i>Diacetyl treatment</i>							
Collagen	29.8	6.8	4.5	22.4	trace	0.0	28.3
<i>Dialdehyde starch treatment</i>							
Collagen	24.8	3.3	1.8	11.2	6.7	22.0	38.8

\* X runs just in advance of ornithine and Y between histidine and ammonia. Both have been calculated in terms of the ninhydrin colour yield of lysine.

\*\* No lysine was present in the guanidated collagen and the peak running here is presumably the reaction product of homoarginine and glyoxal corresponding to X.

#### *Interaction of glyoxal and other dicarbonyl compounds with guanidyl groups*

Treatment with compounds containing adjacent carbonyl groups led to loss of arginine. Some further analyses are given in Table II. With glyoxal and dialdehyde starch two new ninhydrin positive peaks (X and Y) were observed with the 20 × 1 cm IR 120 column, X running just in advance of ornithine and Y running between lysine and ammonia. There were also increases in ammonia. With methylglyoxal only X was produced and with diacetyl only a trace of X. (cf. YANKEELUV *et al.*<sup>27</sup> whose results suggest the formation of ornithine on hydrolysis of salmine treated with diacetyl).

Reaction of Z-arginine and arginine with glyoxal at pH 8.0 followed by hydrolysis in 6 M HCl gave similar elution patterns showing decrease in arginine and peaks corresponding to X and Y. Omission of the reaction period at pH 8.0 had no effect, indicating that the reaction leading to loss of arginine occurs during hydrolysis (Table III). In view of the work of KING<sup>28</sup> on the formation of pyrimidyl ornithine from arginine and malonaldehyde under acid conditions, arginine was reacted with glyoxal in 12 M HCl for 16 h. Ion-exchange chromatography on IR 120 showed that all the arginine had reacted with the formation primarily of X, together with small amounts of Y, ammonia and an additional ninhydrin positive compound A emerging almost with the buffer front (Fig. 4). After heating in 6 M HCl at 105° for 24 h there was a



TABLE III

## REACTION OF GLYOXAL WITH ARGININE AND Z-ARGININE

Ninhydrin reactive peaks were separated on 20 × 1 cm IR 120 column. The results are expressed in mmoles recovered per 100 mmoles arginine reacted.

	A	Orn*	X*	Y	NH <sub>3</sub>	Arg
<i>Z-arginine and glyoxal</i>						
Reacted at pH 8.0, hydrolysed	This part	trace	18	41	15	14
Hydrolysed directly	of run not covered	trace	15	50	12	14
<i>Arginine and glyoxal in 12 M HCl, 24 h</i>						
Unhydrolysed	4	n.d.	30	3	4	trace
Hydrolysed	12	3	16	55	10	36
Unhydrolysed	10	n.d.	42	2	1	
Hydrolysed	14	n.d.	24	approx. 61	3	4
<i>Arginine and glyoxal at pH 2.0</i>						
2 days, not hydrolysed	trace	n.d.	6	0	11	75
14 days, not hydrolysed	trace	n.d.	18	0	4	58
<i>Arginine and methylglyoxal at pH 2.0</i>						
2 days, not hydrolysed	n.d.	n.d.	12	0	2	62
14 days, not hydrolysed	trace	n.d.	30	0	4	46

Abbreviation: n.d., not detected.

\* X runs slightly in advance of ornithine but when a single peak occurs in this position it is not possible to say with certainty whether it is X or ornithine. In all cases where ornithine was added as a marker it gave a shoulder on the peak X. X and Y are calculated in terms of the ninhydrin-colour yield of lysine.

marked decrease in X, increase in Y and A and some release of arginine. Some reaction also occurred at pH 2.0 with glyoxal and methylglyoxal giving primarily X.

The relative amounts of the various compounds varied from one preparation to another and in the absence of information on the ninhydrin-colour yield of the products stoichiometric deductions are unjustified. The most probable interpretation appears to be that X is the primary product and A and Y are breakdown products of this. Either, X breaks down to give 2 or 3 molecules of Y or the colour yield of Y is much greater than X.

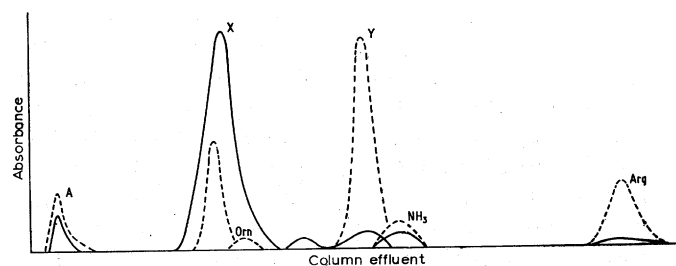


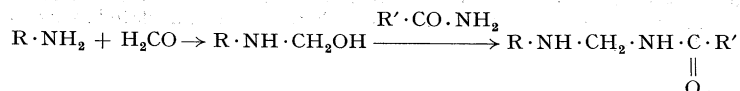
Fig. 4. Separation of arginine-glyoxal reaction products on 20 × 1 cm IR 120 column at 57°. Eluting buffer, 0.38 M citrate (pH 4.46).

## DISCUSSION

It is clear from this work that aldehydes differ markedly in their reactivity towards the amino groups of collagen and also in the extent to which they interact with other groups in the protein. Formaldehyde is unique in that it leads to complete loss of tyrosine. It is very reactive towards amino groups, but the bonds so formed appear to be relatively easily broken. Propionaldehyde, the other monoaldehyde tested, was relatively unreactive.

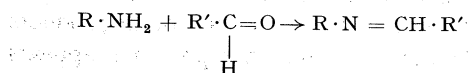
With the dialdehydes several factors appear to be involved, mostly related to stereochemical considerations. For example, the relatively low reactivity of malonaldehyde compared with glyoxal and succinaldehyde is probably due to the readiness with which polymerisation occurs with this aldehyde. The reason for the high reactivity of succinaldehyde and glutaraldehyde and the sharp decrease with adipaldehyde, the next in the series, cannot readily be explained. The marked variation in the effect of pH on reactivity, especially as far as formaldehyde and glutaraldehyde are concerned is also difficult to explain on theoretical grounds. It is hard to believe that variation in the degree to which the carbonyl group dissociates in the two aldehydes can be sufficient to account for such marked difference in reactivity at low pH values.

The nature of the bonds formed also varies with the aldehyde as indicated by lysine recovery and stability of the cross-links introduced. With formaldehyde FRAENKEL-CONRAT and co-workers<sup>2-4</sup> have shown that interaction with amino groups is followed by further condensation with amide and guanidyl groups.

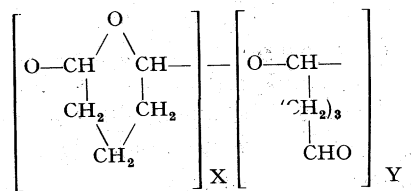


The bonds involved, however, appear to be relatively labile to hot water and acid.

The most probable reaction with the other aldehydes is the formation of Schiff's base type compounds.



Such bonds are, however, generally considered to be relatively labile and would not be expected to withstand acid hydrolysis and lead to loss of lysine residues. It seems probable, therefore, that except with glyoxal, some secondary condensation occurs which leads to stabilisation of these bonds. With glutaraldehyde the evidence suggests that primary reaction is with amino groups to give a product having an ultraviolet absorbing maximum around 265 mμ. Secondary condensation appears to occur during hydrolysis leading to further increases in ultraviolet absorption. The product formed appears to involve only lysine and glutaraldehyde and there is no evidence for the participation of the α-amino group in the reaction. The separation from hydrolysates of a strong ultraviolet absorbing fraction having apparently three glutaraldehyde residues associated with one lysine residue, or multiples thereof, indicates that polymerisation of glutaraldehyde residues must occur. This could be by aldol condensation, though the work of ASO AND AITO<sup>26</sup> suggests that with glutaraldehyde α-oxypyran rings are formed giving tetramers and pentamers containing a pendant aldehyde group.

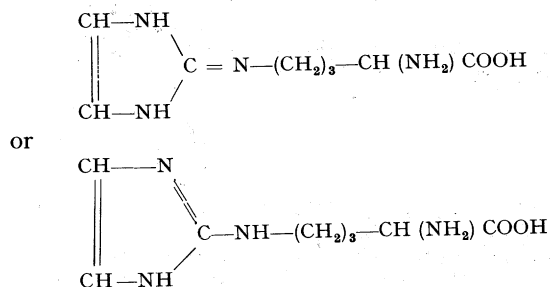


where the ratio of X to Y is about 0.38.

The number of cross-links introduced is apparently influenced by stereochemical factors as well as reactivity and the efficiency of glutaraldehyde and succinaldehyde in this respect is presumably related to the fact that the distances which they or their oligomers can bridge are particularly suitable for the cross-linking *via* amino groups. Variations in the stability of cross-links introduced by different aldehydes, formaldehyde and possibly glyoxal excepted, is probably governed more by the nature of the aldehyde-aldehyde bonds involved than by the differences in the lability of the nitrogen-carbon bonds.

Glyoxal, methylglyoxal, diacetyl and dialdehyde starch were the only carbonyl compounds with which a loss of arginine was observed. Reaction was shown to have occurred during acid hydrolysis and subsequent experiments with glyoxal and arginine showed that complete reaction occurred in 16 h in 12 M HCl at room temperature giving one main reaction product X.

In view of the findings of KING<sup>28</sup> with malonaldehyde and Tori *et al.*<sup>29</sup> with cyclohexanedione the most probable reaction compounds with glyoxal are:



Y and A are probably breakdown products. The relative size of the ninhydrin peaks due to X and Y suggests that breakdown may involve the formation of 2 or more molecules of Y for every one of X lost.

The indications are that glyoxal could be at least as effective as malonaldehyde and diacetyl for the specific modification of the guanidyl group.

The results have some relevance to the reported occurrence of aldehyde groups and Schiff's bases in proteins and their possible involvement in the maturation of collagen. PIEZ and co-workers<sup>11,12</sup>, for example, have demonstrated the conversion of lysine residues to the corresponding  $\epsilon$ -aldehyde and suggest that cross-links are formed by aldol condensation of these aldehydes. TANZER<sup>30</sup> has shown that intra molecular cross-links are stabilised by sodium borohydride and suggests the involvement of Schiff's bases and BAILEY<sup>31</sup> reports similar evidence regarding intermolecular links.

An aldehyde derived from lysine should most nearly resemble propionaldehyde in its reactivity towards amino groups in the collagen. On the basis of the present

results reaction under physiological conditions would be relatively slow. The results, however, also demonstrate the importance of stereochemical considerations, *e.g.* in the reaction of glyoxal and other dicarbonyl compounds with guanidyl residues, and in the protein such factors might well favour the reaction. If cross-links are formed by an aldol condensation between two lysine-derived aldehyde groups the resulting unsaturated aldehyde should be readily reactive towards amino groups.

The present results demonstrate that interaction of aldehydes with lysine residues leads to a marked increase in ultraviolet absorption. In this connection it is interesting to note that ARMSTRONG AND HORSLEY<sup>32</sup> report the isolation of a protein fraction from alkaline hydrolysates of dentine collagen having an absorption maximum at 265 m $\mu$ . Dentine collagen is reported to swell less than that from other sources due, it is suggested, to more extensive cross-linking.

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